Within-ejaculate sperm competition

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Abstract: Sperm competition has been defined by Geoff Parker 50 years ago as the competition between sperm from two or more males over the fertilisation of a set of eggs. Since the publication of his seminal paper, sperm competition has developed into a large field of research, and many aspects are still being discovered. One of the relatively poorly understood aspects is the importance of selection and competition among sperm within the ejaculate of a male. The sheer number of sperm produced in a male's ejaculate suggests that the competition among sibling sperm produced by the same male may be intense. In this review, we summarise Parker's theoretical models generating predictions about the evolution of sperm traits under the control of the diploid male as opposed to the haploid gamete. We review the existing evidence of within-ejaculate competition from a wide range of fields and taxa. We also discuss the conceptual and practical hurdles we have been facing to study within-ejaculate sperm competition, and how novel technologies may help addressing some of the currently open questions.

Keywords: haploid selection, meiotic drive, genetic conflict, multi-level selection

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Sperm in competition

In his landmark paper celebrating its 50th anniversary with this issue, Parker (1970) defined *sperm competition* as the competition between sperm from two or more males over the fertilisation of eggs. The term *sperm competition* therefore by default refers to sperm competition between ejaculates (Parker, 1993). However, because in the vast majority of species, sperm from one male generally outnumber available eggs, the competition among sibling sperm produced by one male is potentially intense (Parker & Begon, 1993; Haig & Bergstrom, 1995). To distinguish between the two forms of sperm competition, we hereafter refer to *between-ejaculate* (between sperm of different males) and *within-ejaculate* (between sperm from one male) competition. While the risk and intensity of between-ejaculate competition vary between mating events and across males and species, within-ejaculate competition may occur during every fertilisation event. The role of between-ejaculate sperm competition in the evolution of sperm and male traits is supported by a large body of evidence (Birkhead & Møller, 1998; Birkhead *et al.*, 2009), whereas the role and importance of within-ejaculate sperm competition for evolutionary processes is less well documented (Joseph & Kirkpatrick, 2004; Immler & Otto, 2018; Immler, 2019). In this review, we focus on the evolutionary role of within-ejaculate competition. We first summarise Parker's theoretical contribution and review theoretical arguments and empirical evidence for within-ejaculate competition.

Parker's models: diploid vs haploid control over sperm phenotype

Among the numerous contributions by Geoff Parker to sperm competition theory (see Parker, this issue for review), two papers, published in parallel, investigated how *diploid* and *haploid* control respectively affect the evolution of sperm characteristics, and how these two scenarios differ (Parker 1993; Parker & Begon 1993). Both studies use game theory to identify Evolutionary Stable Strategies (ESS) for sperm number and sperm size, both influencing fertilisation success, in the context of between-ejaculate competition. All models share the assumption that ejaculate costs are the product of sperm number and size, and that variation in sperm size provides diminishing returns for fertilisation success. Furthermore, ejaculate costs can either trade-off with achieved matings or be fixed, with a trade-off arising between sperm size and number. The main difference between the two sets of models is the assumption that the evolution of sperm size and number are under the control of the diploid male (Parker, 1993) or under the control of the haploid sperm (Parker & Begon, 1993).

The ESS differs substantially between the two sets of models. Under diploid male control, sperm numbers are predicted to increase with the risk of between-ejaculate sperm competition, whereas size shows no effect, unless

density-dependence or survival benefits for larger sperm are invoked (Parker, 1993). When sperm phenotypes are under haploid gametic control, the predicted outcome depends on whether the cost of the mutation favouring the mutant sperm is paid by the male, by sibling sperm carrying the alternative allele, or by sibling sperm carrying the same mutation (Parker & Begon, 1993). Where costs are assumed by the male, size and number mutations (i.e. by diverting resources to increasing sperm size or to increasing rate of cell division and hence sperm number) are predicted to escalate at the expense of achieved number of matings. If the cost is paid by sibling non-mutant sperm, size or number mutations can spread under a size-number trade-off, while mutations that are costly to sibling mutant sperm carrying the same allele do not spread.

One intuitive prediction resulting from the conflict between a male and its sperm is that within-ejaculate competition in species with high risk of sperm competition should be minimised, due to the potential costs to the male (Figure 1A). However, Parker and Begon (1993) showed that even under maximum risk of between-ejaculate sperm competition, conflicts between male and sperm do not disappear (Parker & Begon, 1993). Indeed, theoretical models for the evolution of 'soldier sperm' attacking a rival male's sperm by sacrificing their own fertilisation ability in favour of sibling sperm show that these can only evolve if the control lies with the diploid male (Kura & Nakashima, 2000). A more recent model predicted that alleles favoured in within-ejaculate competition can spread rapidly if they are neutral (or beneficial) to diploid fitness (Ezawa & Innan, 2013). Similarly, another recent model confirmed that haploid selection is maintained even under scenarios of sperm competition, if selection on haploid gametes results in the efficient removal of deleterious mutations from a population (Otto *et al.*, 2015).

Within-ejaculate competition driving sperm evolution

A male shares 50% of its alleles with all its sperm carrying a full set of haploid chromosomes. Sibling sperm are on average also 50% related to one another but this may vary depending on the rate of segregation, recombination and the heterozygosity of an organism. This situation could be compared to scenarios of parent-offspring conflict, where individual offspring are selected to be selfish at the cost of parental fitness (Trivers, 1974; Godfray, 1995). Sperm traits that have been thought to mediate possible conflicts in favour of the diploid male include a densely re-packaged DNA and suppression of post-meiotic transcription, cytoplasmic bridges linking haploid spermatids with each other for efficient sharing of transcripts, and control of haploid gametes through

diploid-expressed RNA or seminal fluid (Frank, 1995; Hosken & Hodgson, 2014; Ågren *et al.*, 2019). For sperm traits to evolve through within-ejaculate selection, three criteria for evolution to occur in general need to be met: 1) sperm need to exhibit phenotypic variation 2) sperm phenotypes must be heritable and 3) sperm phenotypes need to affect fitness (Lewontin, 1970). We only briefly discuss evidence for each of these, as all three have been discussed earlier in extensive reviews (e.g. Holt & Van Look, 2004; Immler & Otto, 2018; Immler, 2019).

Phenotypic variation among sibling sperm is well documented, but whether this variation arises for accidental or adaptive reasons is still not fully understood (Holt & Van Look, 2004; Pitnick *et al.*, 2009; Higginson & Pitnick, 2011). Potential, non-mutually exclusive explanations for phenotypic variation include sperm production errors (e.g. Stewart *et al.*, 2016), strategic variation for bet-hedging (Till-Bottraud *et al.*, 2005), distinct casts of sperm phenotypes (Kura & Nakashima, 2000; Pizzari & Foster, 2008), and manifestation of haploid interests (Parker & Begon, 1993; Immler, 2019). Observed patterns are often compatible with several of these hypotheses. For example, the observation that within-ejaculate phenotypic variation correlates negatively with the level of sperm competition (e.g. Immler *et al.*, 2008; Lifjeld *et al.*, 2010; but see Sharma *et al.*, 2013) could be explained by stabilising selection on optimal sperm phenotypes under increased risk of sperm competition (Bernasconi & Hellriegel, 2005), but also by a reduction of the haploid-diploid conflict with increasing importance of between-ejaculate competition in species with high sperm competition risk (Parker & Begon, 1993; see Fitzpatrick & Baer, 2011 for a rare exception).

In order for phenotypic variation to be heritable, sperm phenotypes need to at least partially reflect the haploid sperm genotype (Figure 2). It was long thought that genome condensation in developing sperm would largely silence gene expression (e.g. Steger, 1999), and that cytoplasmic bridges between spermatids would essentially homogenise any potential remaining differences (Dadoune *et al.*, 2004). The very fact that sperm are so small may be related to avoiding selfish genetic (cytoplasmic) elements acting in sperm (Randerson & Hurst, 1999), and the evolution of other aspects of spermatogenesis may have been fuelled by intragenomic conflict with selfish genetic elements (Verspoor et al., this issue). Nevertheless, there is now ample evidence for postmeiotic transcription (Dadoune *et al.*, 2004; Joseph & Kirkpatrick, 2004; Vibranovski *et al.*, 2010; Ren *et al.*, 2017), and many transcripts are not equally shared via cytoplasmic bridges (Zheng *et al.*, 2001; Ventelä *et al.*, 2003; Véron *et al.*, 2009; Bhutani *et al.*, 2019). Ways for males to control the effects of haploid expressed genes and prevent within-ejaculate competition are for example by provisioning sperm with diploid-derived RNA (Hosken &

Hodgson, 2014) or by affecting sperm via the composition of the seminal fluid (Pizzari & Parker, 2009; Perry *et al.*, 2013).

Finally, even if sperm are able to express their genotype, this expression needs to result in a phenotypic difference that influences their chance of winning fertilisations. Although it is conceivable and intuitive that different phenotypes would have different chances of fertilising ova, this connection is not always explicitly established. Empirical evidence for within-ejaculate competition with fitness consequences and thus evolutionary potential comes from some meiotic drivers (Zheng *et al.*, 2001; Véron *et al.*, 2009; Rathje *et al.*, 2019). Outside of these (perhaps extreme) examples, indications that within-ejaculate competition has evolutionary potential comes from studies linking within-ejaculate sperm selection to offspring fitness (Immler *et al.*, 2014; Alavioon *et al.*, 2017; Pérez-Cerezales *et al.*, 2018), though the underlying mechanisms remain somewhat elusive.

Potential costs and benefits of within-ejaculate competition

In most species, sperm are produced in vast numbers, but only very few of them end up fertilising eggs, potentially resulting in strong selection for the 'best' sperm (e.g. Manning & Chamberlain, 1994; Ezawa & Innan, 2013). Novel genotypes can be generated through *de novo* mutations, recombination and segregation events, and natural selection for the best sperm may act in two ways: purifying selection removing deleterious mutations and genotypes (Figure 1C), and positive selection for optimal genotypes and beneficial mutations (Manning & Chamberlain, 1994; reviewed in Immler & Otto, 2018; Immler, 2019; Figure 1D). Unlike in diploid selection, where a potential masking effect can 'hide' recessive alleles (Crow & Kimura, 1970; Orr & Otto, 1994), alleles expressed in a haploid genome means that they may be exposed to selection, rendering haploid selection much more efficient. If there is a positive correlation between a haplotype's performance in gametic selection, and its fitness effects in the diploid phase, selection at the haploid gametic stage could offer a cheap and efficient way of trying out new allelic combinations (Immler, 2019).

As mentioned above, under gametic control over sperm traits, mutant sperm gain within-ejaculate competitiveness either at the expense of the diploid male, their sibling sperm with an alternative allele, or their sibling sperm with the same mutant allele (Parker & Begon 1993). Empirical data from sperm competitiveness of males with meiotic drivers suggest that a combination of all three scenarios can occur (Price *et al.*, 2008;

Sutter & Lindholm, 2015). When mutant sperm gain a fitness advantage at the expense of sibling sperm carrying an alternative allele, intra-locus conflict will arise (Figure 1B). Moreover, if the haploid mutant allele has a deleterious effect in diploids, the conflict can extend to the rest of the genome, and selection on the diploid genome should favour suppression of the selfish mutant allele (Leigh, 1977). Thus, if within-ejaculate competition is costly for the diploid male, lineages that can silence this competition are expected to outcompete lineages that do not (Prout *et al.*, 1973; Leigh, 1977; Verspoor, Price & Wedell, this issue). However, the efficiency of haploid selection allows alleles with deleterious effects in the diploid organism to remain in a population (Immler *et al.*, 2012). In fact, even alleles that are recessive lethal to the diploid organism can increase in frequency if their effects are sufficiently beneficial for within-ejaculate competition (Bruck, 1957). However, because these alleles are recessive lethal, they cannot go to fixation, and a stable polymorphism prevents the population from reaching its fitness maximum (Lindholm *et al.*, 2016).

Evidence for and against within-ejaculate sperm competition

While evidence for evolution through between-ejaculate competition has been shown across taxa in a large body of experimental and comparative studies (Birkhead & Møller, 1998), the evidence for evolution through within-ejaculate competition is much scarcer. Part of the reason for the paucity of studies is the technical difficulty to show a process occurring between cells during an often very limited amount of time. In addition, such competition can often only be monitored inside the female reproductive tract or an aquatic environment, making the tracking of individual sperm virtually impossible. An additional reason is the aforementioned long-standing assumption that genetic differences among haploid sperm contribute little if anything to the phenotypic variation (reviewed in Joseph & Kirkpatrick, 2004). This view has recently been challenged as the evidence for gene expression at the post-meiotic haploid stage is steadily increasing (e.g. Bhutani *et al.*, 2019; Raices *et al.*, 2019; Rathje *et al.*, 2019; for reviews see Joseph & Kirkpatrick, 2004; Immler & Otto, 2018; Immler, 2019). While haploid gene expression is not the only way haploid selection among sperm can operate, it certainly increases the opportunity for evolution through within-ejaculate competition.

The most convincing evidence for within-ejaculate competition comes from studies in a range of plants. Haploid gene expression in pollen is well established, and experimental evidence suggests that competition among pollen from the same male improves the fitness of the resulting seedlings (Niesenbaum, 1999). In addition, two studies in the grand shepherd's purse *Capsella grandiflora* (an extreme outcrossing species) and the thale cress

Arabidopsis thaliana (an extreme selfing species) showed increased levels of purifying and positive selection among genes expressed at the haploid stage (Arunkumar *et al.*, 2013; Gossmann *et al.*, 2014). The fact that a similar genomic signature is found in species with very contrasting levels of outcrossing suggests that the outcome of haploid selection may be aligned with diploid fitness interests in these species. In animals, several recent studies have provided evidence for selection and competition among haploid sperm. In the zebrafish *Danio rerio*, pools of longer-lived sperm exhibited allelic differences across the entire genome compared to shorter-lived and immotile sperm (Alavioon *et al.*, 2017). Similarly, a link between marker alleles and sperm phenotypes has been reported in a male hybrid between two *Astyanax* cavefish (Borowsky *et al.*, 2018). In that study, sperm were exposed to a dye challenge, resulting in the grouping of sperm phenotypes sharing similar allelic contents. In mammals, the most direct evidence for a link between sperm genotype and sperm phenotype comes from studies in the house mouse *Mus musculus*, where X- and Y-bearing sperm differ in motility (Rathje *et al.*, 2019) that are not driven by size differences as for example suggested in human sperm (Cui, 1997). Sperm sexing based on membrane proteins in mice has been proposed as an efficient mechanism to determine offspring sex in domestic cattle (Umehara et al., 2019), though it is questionable whether this would translate from *in vitro* into *in vivo* applications (Navarro-Costa et al., 2020). In domestic bull, X and Y-sperm differ by nine nuclear DNA coded proteins (Scott *et al.*, 2018). The different survival of X- and Y-sperm in the female reproductive tract of mammals including humans has been suggested several times, but these observations are currently still anecdotal. The recent findings of a wide range of genes showing biased gene expression across haploid spermatids in house mice and the cynomolgus primate *Macaca fascicularis*, with the same genes showing signs of directional selection in primate and human populations (Bhutani *et al.*, 2019), suggest that a rather large number of genes could actually be involved in determining sperm phenotypes. Again, the function of these genes and their effect on sperm phenotype is currently unclear.

Some indirect evidence for the potential of within-ejaculate competition may come from the fact that many species with a high risk of sperm competition produce dimorphic sperm, which vary not only in their morphology and size but also their genetic content (Till-Bottraud *et al.*, 2005; Pitnick *et al.*, 2009). Often one of the two sperm morphotypes shows a partial or complete lack of DNA (apyrene sperm), rendering them incapable of successfully fertilising eggs (Snook & Karr, 1998). Apyrene sperm appear to have the sole purpose of aiding sperm competition processes by occupying space inside the female sperm storage organs, and/or of protecting sibling sperm from the hostile environment inside the female reproductive tract (Holman & Snook, 2008). The

196 lack of DNA in apyrene sperm results in the effective removal of any genetic conflict with their eupyrene sibling
197 sperm and could be seen as an efficient way to allow for sperm cooperation. However, sperm cooperation has
198 been suggested in other taxa not exhibiting any obvious sperm dimorphism. In the New World opossum
199 *Didelphis virginiana* for example, two sperm joined at their heads are necessary to reach the site of fertilisation,
200 but only one sperm will be able to fertilise the egg as the other one has to undergo an acrosome reaction to
201 separate from its sibling sperm (Rodger & Bedford, 1982). A similar process of acrosome reaction is necessary
202 for sperm in a 'train' to dislocate from each other in the European wood mouse *Apodemus sylvaticus* (Moore *et*
203 *al.*, 2002). A remaining question at this point is whether sperm that undergo acrosome reaction differ genetically
204 from those which get to fertilise the egg, or whether this is a process of pure chance. More generally, the question
205 about whether these observations are a form of cooperation in the evolutionary sense remains controversial
206 (Immler *et al.*, 2007; Firman & Simmons, 2009; Fisher & Hoekstra, 2010; Varea-Sanchez *et al.*, 2016). While
207 sperm can preferentially cooperate with sibling sperm from the same male when mixed with a competitor male's
208 sperm in the deer mouse *Peromyscus maniculatus* (Fisher & Hoekstra, 2010), how the roles are divided within
209 an ejaculate is currently unknown (Moore & Moore, 2002). General predictions are that cooperation among
210 sperm could dynamically arise through male enforcement and be eroded by sperm selfishness (Kura &
211 Nakashima, 2000; Immler, 2008; Pizzari & Foster, 2008; Hosken & Hodgson, 2014).
212
213 As discussed above, part of the dearth of evidence for within-ejaculate sperm competition may have been caused
214 by the lack of technologies, which are now becoming available. Another reason for the scarcity of evidence
215 could be that a *de novo* mutation that is beneficial for the haploid phase would go to fixation relatively rapidly
216 (Ezawa & Innan, 2013; Figure 1D). This is particularly true if it has no effects or a positive effect at the diploid
217 life stage. The detection of such mutations would be difficult, as these would have to be tracked before fixation.
218 The only way to maintain a genetic polymorphism is, if such haploid-beneficial mutations have a negative, partly
219 recessive effect inducing fitness cost to the diploid phase, which results in balancing selection (Immler *et al.*,
220 2012). Such situations are well-described in meiotic drivers, where selfish benefits in the (typically male)
221 haploid phase are counterbalanced by costs in the diploid phase (Lindholm *et al.*, 2016; see also Verspoor, Price
222 & Wedell, this issue).
223
224 Finally, it is possible that some sperm traits are under haploid control while others are under diploid control.
225 The evidence for diploid control over morphological sperm traits and sperm total length in particular (usually

largely determined by the length of the flagellum) is convincing. An explicit test of diploid versus haploid control over the evolution of sperm length was performed in a study on *Drosophila* fruit fly lines that had been selected for long and short sperm, respectively (Pitnick *et al.*, 2009a). F1 crosses between these lines were performed with the prediction that if sperm length was at least partially determined by the haploid genotype, crosses between the lines should show increased variation in sperm length compared to the two parental strains. However, the offspring from crosses between the two lines showed intermediate lengths of sperm and no increased variation compared to the parental lines. In contrast, a recent study using a similar approach of crossing two *Astyanax* cavefish species to generate increased heterozygosity in the F1 offspring reported increased variation in sperm swimming velocity (Borowsky *et al.*, 2019). Many possible biological mechanisms can explain the divergent observations between these two studies, and we can currently only speculate as to which are true.

The future of within-ejaculate sperm competition

The past few years have provided some exciting new insights into the role and importance of within-ejaculate competition. However, we are only at the beginning of understanding what is really happening at this stage of the life cycle, and key questions currently remain unanswered. Based on the topics we reviewed in the previous sections, we discuss some of the currently open questions and how it may be possible to address them.

The first set of questions evolves around identifying the 'best' sperm in an ejaculate: Is there a 'best' sperm and if so, which one is it? Which traits contribute to the success of a sperm in within-ejaculate sperm competition? Do these depend on environmental conditions? These questions are difficult to answer at the moment and opinions are divided. Evidence suggesting that the differences among sperm/pollen in how they fertilise eggs is at least partly genetically determined is quite strong (Niesenbaum, 1999; Alavioon *et al.*, 2017). However, the exact genomic mechanisms are currently not known. The finding of increased purifying selection in haploid-expressed genes in flowering plants and mammals suggests that competition and selection among sibling sperm may serve as a potential quality check allowing to separate the 'wheat from the chaff'. It appears that in both pollen and sperm, physiological performance rather than morphology ultimately determines differences among sibling gametes. A methodological part of the challenge is understanding which sperm characteristics are important for fertilisation potential, particularly in internal fertilisers. Morphological variation in sperm length or shape are relatively easy to measure, and can be a good proxy for fertilisation success, at least when comparing

between males (for reviews see Snook, 2005; Lüpold & Pitnick, 2018). The current literature shows a bias towards detailed studies of morphology, but more recent developments for example in microfluidics (Knowlton *et al.*, 2015), single-cell sequencing (Wang *et al.*, 2012), and the 'omics revolution (Baker, 2011) allow more detailed assays of individual sperm performance *in vitro* and *in vivo*, and a comparison of the two (e.g. Holt *et al.*, 2010; reviewed in Hook & Fisher, 2020). A further possible challenge is that the traits under selection may not always be the same if environmental conditions vary during fertilisation—which they often do (Reinhardt *et al.*, 2015). Moreover, the fertilisation environment is partly determined by females, arguably more so in internal fertilisers (Birkhead *et al.*, 1993). In any case, heterogeneity in environments and coevolutionary dynamics between the sexes make understanding the complexity of sperm evolution a formidable challenge (Reinhardt *et al.*, 2015).

A second question is about whether variation—both genetic and phenotypic—among sibling sperm is systematic as opposed to arising from simple 'production errors'. Understanding the role of purifying and directional selection, as well as understanding which sperm traits are under diploid and which are under haploid control are the future challenges we are facing. Technologies such as single-cell sequencing and more generally single-cell 'omics will help addressing these questions.

A third question is about the methods and species that are best suited for the study of within-ejaculate sperm competition. The ability to generate a natural fertilisation environment *in vitro* is key to understand the biologically relevant sperm traits under selection (Lüpold & Pitnick, 2018; Hook & Fisher, 2020). An alternative route is to employ ever-improving technology such as micro-filming *in situ*, allowing the tracking of sperm within the female reproductive tract (Manier et al., 2010). Alternatively, we can use sequencing and genotyping technologies to assess genetic similarities and differences among offspring sired by varying sperm phenotypes selected for specific traits. In this case, species producing large numbers of offspring may be beneficial, but this can be alleviated if offspring from many families are genotyped.

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Figures

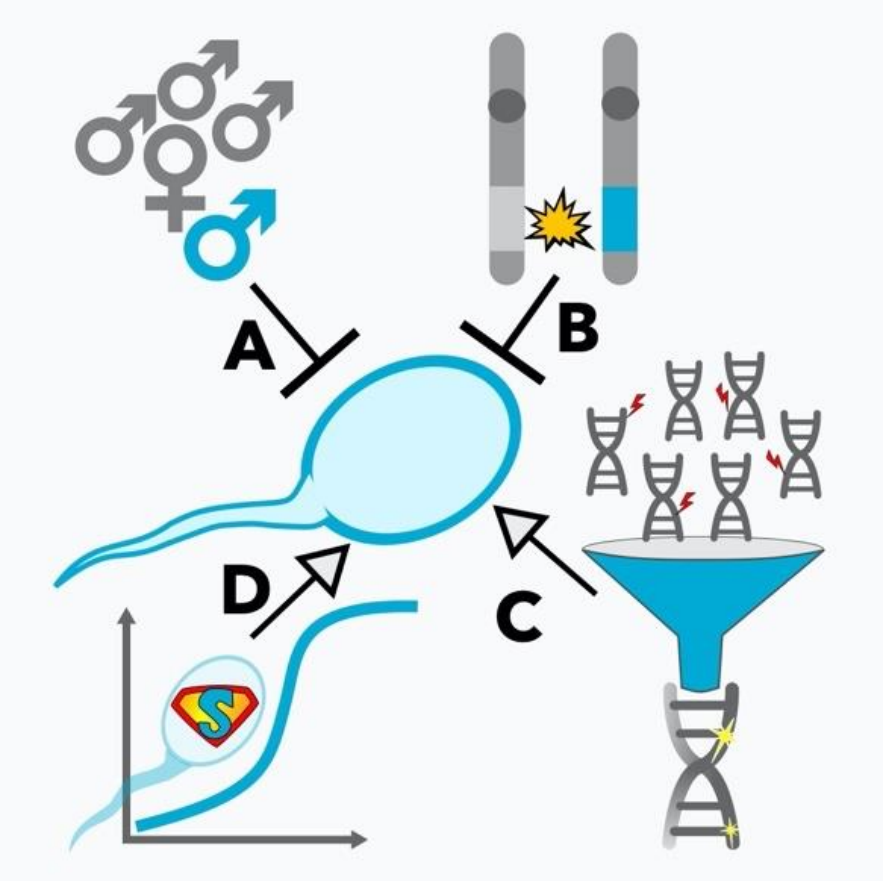

Figure 1: Factors that are expected to hinder or favour within-ejaculate sperm competition. (A) Between-ejaculate sperm competition is predicted to reduce the importance of within-ejaculate sperm competition. (B) While mutant alleles with a haploid advantage may favour within-ejaculate competition, alternative alleles paying the cost of the mutant allele should suppress within-ejaculate sperm competition. If mutant alleles favoured in within-ejaculate sperm competition have deleterious effects on diploid fitness, the entire diploid genome is under selection to evolve a resistance mechanism to suppress the mutant allele. (C) If efficient purifying selection via haploid selection is possible, selection should favour within-ejaculate sperm competition. (D) A similar situation occurs if mutations are beneficial for within-ejaculate sperm competition *and* diploid fitness. Such alleles are expected to quickly sweep to fixation and will be hard to trace.

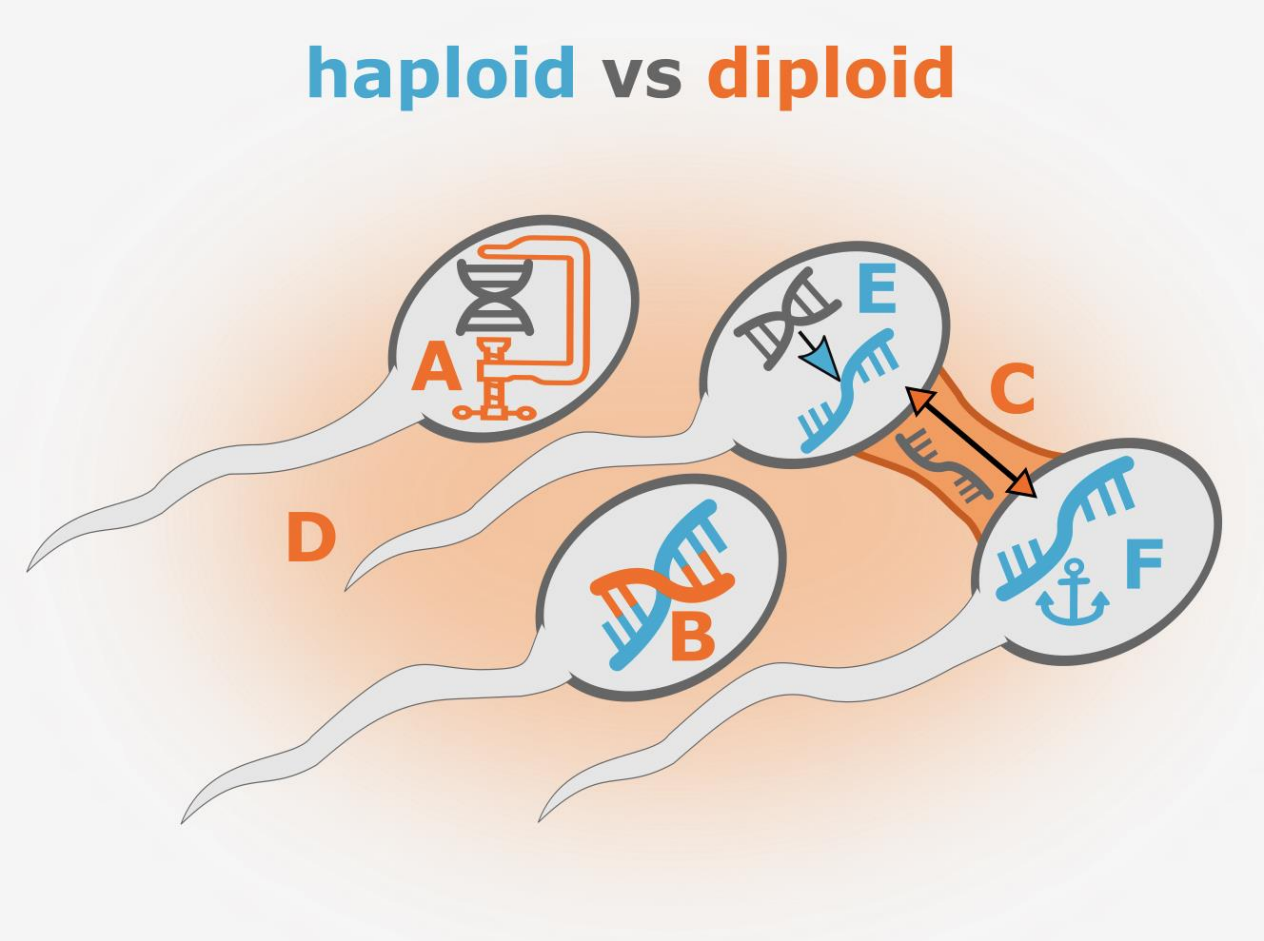

Figure 2: Biological mechanisms facilitating versus suppressing within-ejaculate sperm competition. Schematic representation of conflict between haploid sperm and the diploid organism over control of sperm phenotype. Mechanisms by which sperm may facilitate (blue) and the diploid organism may hamper (orange) haploid control, respectively, are shown. The diploid organism may attempt to silence haploid gene expression through (A) DNA condensation or (B) RNA interference, and may eliminate differences between sperm through (C) sharing of haploid-expressed RNA and proteins via cytoplasmic bridges or through (D) control over sperm phenotype by seminal fluid. Sperm may attempt (E) haploid transcription/translation, and (F) haploid retention of RNA and proteins to avoid homogenisation among sibling sperm.